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Energetic Effect of Adenosine on *Drosophila* Imaginal Disc Cells

Master Thesis
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Anotace:
The effects of extracellular adenosine on *Drosophila* imaginal disc cell line Cl.8+ were examined. The Cl.8+ cells were grown in the complete medium and tested for viability, growth rate, ATP production and O_2 consumption in the presence or absence of adenosine. It was discovered that extracellular adenosine is a key regulator of the energetics of Cl.8+ cells.

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Prohlašuji, že jsem tuto magisterskou diplomovou práci vypracovala samostatně pouze s použitím uvedené literatury.

Kateřina Šandová

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1 INTRODUCTION

1.1 General Introduction

Adenosine is a multifunctional nucleoside that is most important as an intracellular precursor of nucleic acids and ATP, but it is also a key extracellular signaling molecule affecting a variety of cellular processes. The interest in adenosine as a signaling molecule goes back at least as far as 1929. It has been known since the early study of Drury and Szent-Gyorgyi that adenosine injection increased coronary blood flow, reduced heart beat rate and lowered systemic blood pressure (Drury and Szent-Gyorgyi, 1929). In later studies, the extracellular adenosine was also implicated in the regulation of immune and neural functions, as well as in the responses to stress and hypoxia.

Adenosine affects cells via binding to specific receptors or is transferred across the cell membrane via adenosine transporters. The existence of four adenosine receptors in mammals with different functions but overlapping patterns of expression, together with a number of adenosine transporters of two types and the pervasiveness of adenosine-mediated physiological events, pose difficult questions in efforts to design pharmacological and biochemical interventions (Nyce, 1999).

The analyses of complex signaling pathways are often successfully performed by genetic dissection in *Drosophila* and other model organisms. The number of genes involved in the *Drosophila* adenosine-signaling pathway including adenosine receptor and transporter is lower than in mammals. In our laboratory we have characterized several important genes involved in the *Drosophila* adenosine signaling, including the ADGF family of adenosine deaminases and adenosine receptor. *Drosophila* imaginal disc cells (Cl.8+) were shown to be very sensitive to extracellular adenosine and were chosen as a model for these studies. My research project attempts to answer some questions regarding adenosine cytotoxicity and the role of adenosine in cellular energetics. I examined the influence of extracellular adenosine on the level of ATP and aerobic respiration in Cl.8+ cells. The experiments also include investigations of adenosine effects on cell growth and viability of Cl.8+ cells in complete medium.

1.2 Adenosine

Adenosine is a nucleoside formed from adenine attached to a ribose ring via a β-N_9-glycosidic bond (Figure I). It is recognized as an interesting signaling molecule with a complex mode of action. Adenosine serves as a precursor connected with numerous biochemical processes. As adenosine triphosphate (ATP) and adenosine diphosphate (ADP) it is highly involved both in energy transfer and in extracellular signaling. It is also an essential component of second messenger molecule-cyclic adenosine monophosphate (cAMP).

Adenosine is a significant extracellular signaling molecule involved in regulation of various physiological and pathological processes. It is involved in the regulation of homeostasis, it is well known for its crucial role in preconditioning, which is a phenomenon intensively studied on cardiovascular system of mammals (Headrick *et al*., 2003; Stein *et al*., 2004). It plays a role in oxidative stress (Zhang *et al*., 2005), cell division or cell death (Wang & Ren 2006; Ohkubo *et al*., 2007), immune response and inflammation (Cronstein *et al.,* 1994; Bours *et al*., 2006; Desrosiers *et al*., 2007), sleep and waking cycles (Blanco-Centurion *et al.*, 2006) and many others.

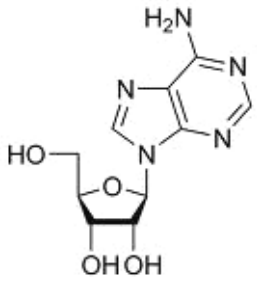


FIGURE I. Structural formula of adenosine

Adenosine is mainly formed by the dephosphorylation of intra- or extracellular adenine nucleotides (ATP, ADP or AMP), catalyzed by 5' nucleotidase (Zimmermann, 2000; Latini & Pedata, 2001). But there are also other independent sources, such as degradation of nucleotides from apoptotic cells or hydrolysis of S-adenosyl homocystein (Hershfield *et al*., 1985; Deussen, 1989).

The levels of adenosine in cells and tissue fluids are in the nanomolar range under physiological conditions (the range is estimated between ten and a few hundred nanomolar), but rise substantially in various forms of cellular distress (Fredholm, 2007). Two enzymes play a key role in the maintaining of low physiological levels of adenosine: adenosine kinase (AK) and adenosine deaminase (ADA). The AK performs the conversion of adenosine by its phosphorylation to AMP, whereas ADA catalyzes the irreversible deamination of adenosine and deoxyadenosine to inosine and deoxyinosine, respectively. Deoxyadenosine is a cytotoxic metabolite released by various cell populations that undergo programmed cell death (Hershfield & Mitchell, 2001).

1.3 ADA and ADGF

Mammalian ADAs are mainly cytosolic proteins. There are also some studies on so-called ecto-ADA, which is localized on the cell surface as well and is suggested to regulate extracellular adenosine levels (Franco *et al*., 1990). ADA is a critically important enzyme for human immunity because its congenital absence causes severe combined immunodeficiency disease (SCID) (Giblett *et al*., 1972; Hershfield, 2005). This disease is associated with the depletion of T and B-lymphocytes and natural killer cells, resulting in impaired cellular immunity and decreased production of immunoglobulins (Buckley *et al*., 1997). The accumulation of adenosine and deoxyadenosine and sensitivity of immature lymphoid cells to the toxic effect of these substrates lead to the consequent immunodeficiency (Hershfield & Mitchell, 2001). ADA is not the only adenosine deaminase in mammalian cells. Riazi *et al*. (2000) identified a gene named CECR1 (Cat eye syndrome critical region 1), which product is related to but distinct from classical ADAs.

The level of extracellular adenosine in *Drosophila* is regulated by a protein family called ADGF (Adenosine deaminase-related growth factors) described by Zurovec *et al*. (2002). It is encoded by six genes with sequence similarity to the CECR1 protein and to adenosine deaminases. At least two of the products (ADGF-A and ADGF-D) exhibit a strong ADA activity (Zurovec *et al*., 2002). It was proved by Dolezal *et al*. (2005) that ADGF-A is essential for insect growth and development because the disruption of this gene in *Drosophila* is larval lethal. Double mutants in ADGF C+D genes are lethal in late pupa and remaining adult survivors are lethargic and die within one week (Gazi, 2004).

1.4 Equilibrative Nucleoside Transporters (ENTs)

Adenosine is a hydrophilic molecule and both passive and active transport systems are required for the flux across the cell membrane. There are equilibrative nucleoside transporters (ENTs) (Belt, 1983) that function mainly by facilitated diffusion, and concentrative nucleoside transporters (CNTs) (Thorn & Jarvis, 1996), in which the transport of a nucleoside is driven by a co-transport of Na+ down its concentration gradient (Sankar *et al*., 2002).

The equilibrative transporter system mediates nucleoside transfer in both directions depending on the nucleoside concentration gradient across the plasma membrane. The ENT proteins have been reported in a variety of organisms. Four equilibrative transporters (ENT1,

ENT2, ENT3 and ENT4) were identified in mammalian cells. ENTs are widely distributed among various cell types, but the number of molecules of each nucleoside transporter depends on the cell and tissue type. All these transporters are able to transport adenosine, but they have different capacity and specificity to transport other nucleosides, nucleobases or their analogues widely used in pharmacology (Kong *et al*., 2004). The hENT1 is present in a variety of tissues, including heart, liver, kidney, brain and others. The highest expression of hENT2 was observed in skeletal muscle (Griffiths *et al*., 1997). The hENT3 has also been detected in several tissues but it was shown to be lysosomal transporter localized intracellularly (Baldwin, 2004). The hENT4 has been identified recently, it is expressed in the heart but it seems to have its optimal transporting activity only at acidic pH (Barnes *et al*. 2006). Transporters differ among each other in susceptibility to inhibition by specific compounds. The hENT1 and hENT3 are sensitive to NBTI (nitrobenzylthioinosine) and coronary vasodilator dipyridamole whereas the hENT2 is much less susceptible to these compounds. However, these investigations differ among species (Yao *et al*., 1997). Adenosine transporters are implicated in the heart protection together with adenosine receptors. Formerly mentioned dipyridamole is effective in blocking the efflux of adenosine into the cell and therefore helps the receptor to be efficiently exposed to adenosine (Meester *et al*., 1998).

In addition, there are temporal, spatial and quantitative variations in ENT expression both within and between individuals suggesting complex transcriptional regulation, which, in a clinical setting, may influence drug efficiency (Pennycooke *et al*., 2001).

The ENT proteins were also described in invertebrate models. In *Drosophila* a comparative genomic analysis revealed the existence of three ENT proteins. DmENT1, DmENT2 and DmENT3 were identified by Sankar *et al*. (2002). Although the sequence identity in comparison to mammalian ENTs is limited, they are clearly unified by common structural motifs (Sankar *et al*., 2002). In *Drosophila* imaginal disc cells (Cl.8+) a relative levels of ENT gene expression were detected (Zurovec, unpublished results). According to this analysis, it was proved that ENT2 gene exhibits the highest intensity of expression of the three genes. In addition, in Cl.8+ cells, the high ENT2 expression is considered to be a crucial factor in the apoptosis induced by elevated adenosine concentration (Steinbauerova, 2005). Studies *in vivo* reveal that adenosine transporters might be involved in regulation of some neural processes such as learning and memory in *Drosophila* (Boulianne, unpublished results).

1.5 Adenosine Receptors (AdoRs)

Adenosine receptors belong to a family of G protein-coupled receptors (GPCR). All GPCRs are composed of 7 hydrophobic regions, which span the plasmatic membrane (Palmer & Stiles, 1995). Mammalian adenosine receptors are ubiquitously expressed in a wide variety of tissues. They are classified into four types: A1, A2a, A2b and A3 (Fredholm *et al*., 2000). Their function in signaling is connected with different downstream pathways including modulation of intracellular adenosine 3', 5'-cyclic monophosphate (cAMP) levels: A1 and A3 inhibit adenylate cyclase, whereas A2a and A2b stimulate this enzyme (van Calker *et al*., 1979; Londos *et al*., 1980).

Adenosine receptor knockout mice were generated in order to get some insights into the physiology and pathophysiology of different adenosine receptors. Using the A2a receptor knockout mice Ledent *et al.* (1997) found, that A2a receptors mediate pain signal via peripheral sites, inhibit platelet aggregation and regulate blood pressure. The mice with targeted disruption of A1 receptor show increased anxiety and are hyperalgesic (Johansson *et al*., 2001). The A3 receptor knockouts show a decrease in vascular permeability (Tilley *et al*., 2000) and mast cell degranulation (Salvatore *et al*., 2000). The A2b receptors mediate the release of allergic mediators from mast cells (Auchampach *et al*., 1997).

The function of particular mammalian AdoRs can differ among species and even among various tissues (Fredholm *et al*., 2001). Although there is an extensive effort to study all adenosine receptors in pharmacological and biochemical manner, it is hard to make some clear conclusions. Each receptor has different function, but patterns of their expression often overlap. Moreover, it was shown, that there is a crosstalk among various GPCR receptors, thus the molecular dissection of AdoR signaling becomes more difficult (Fredholm *et al*., 2000).

The understanding of extracellular adenosine effects can be advanced by the use of less complicated *Drosophila* model. The first (and so far the only one known) invertebrate adenosine receptor was investigated in *Drosophila* (encoded by the gene CG9753) (Dolezalova, 2004). It was shown, that adenosine stimulation of CHO cells (Chinese hamster ovary cells) carrying transiently expressed CG9753 led to a dose-dependent increase in intracellular cAMP and calcium (Dolezalova *et al*, 2007). In addition, a loss-of-function mutation in the CG9753 gene (AdoR) was also generated, however, this mutant does not have any obvious phenotype. Interestingly, it was shown, that the AdoR/ADGF-A double mutants

exhibit a less extreme phenotype than the ADGF-A null mutant (Dolezal *et al*., 2005). This confirms that the two genes are functionally related. Nevertheless, some phenotypic features of ADGF-A mutation were not rescued by AdoR knockout (Dolezal, 2005). Therefore the adenosine transport might also influence the phenotype of the ADGF-A mutant.

1.6 Adenosine Phosphates and AMP-Activated Protein Kinase (AMPK)

Adenosine is also an essential part of nucleoside phosphates (ATP, ADP, AMP).

Adenosine triphosphate (ATP) is the most important molecule for capturing and transferring the energy in living organisms. It is considered as a universal "currency" of chemical energy. The energy is released during hydrolysis of the terminal phosphoanhydride bond. ATP is generated from adenosine diphosphate (ADP) and inorganic phosphate (HPO_4^{2-}). This endergonic reaction, which is the reverse of ATP hydrolysis, requires an input of 7.3 kcal/mol. (Lodish *et al.* 2004) The energy to drive this reaction is produced mainly by aerobic oxidation, which occurs in mitochondria of almost all cells.

Living cells normally maintain a high ratio of ATP/ADP (typically around 10:1), which is far from the equilibrium ratio for ATP hydrolysis under cellular conditions. This represents a store of energy that can be used to drive energy-requiring processes. Catabolism converts ADP to ATP, whereas most other cellular processes require ATP hydrolysis and tend to decrease the ratio. Most cells manage to maintain their ATP:ADP ratio within fairly narrow limits, which indicates, that the rate of ATP synthesis exactly matches the rate of ATP consumption (Hardie, 2004). Since the reaction, catalyzed by adenylate kinase ($2ADP \leftrightarrow ATP+AMP$) is maintained close to equilibrium, AMP/ATP ratio is much more sensitive indicator of cellular energy status (Hardie & Hawley, 2001).

A crucial role in the maintaining of this balance is played by AMP-activated protein kinase (AMPK). The AMPK is sensitive to the change in the AMP/ATP ratio and serves as a metabolic sensor of eukaryotic cells (Hardie & Hawley, 2001). The AMPK is activated in response to the ATP depletion, associated with an increase in the cellular AMP:ATP ratio. The 5'-AMP activates the AMPK system by three independent mechanisms: (1.) allosteric activation of the enzyme by binding to AMP, (2.) allosteric activation by the upstream AMPK kinase (LKB-1), which increases the AMPK activity by its phosphorylation and (3.) inhibition of dephosphorylation by protein phosphatases (Hardie *et al*., 1999). The AMP-binding sites

also bind ATP in a manner that is mutually exclusive with AMP. The affinity of these sites for the ATP is lower than for the AMP (Scott *et al*., 2004) and since the ATP binding does not cause any of the three activating effects of AMP, high concentrations of ATP inhibit activation of AMPK by antagonizing binding of AMP (Hardie *et al*., 2006). This triplicate mechanism of AMPK activation results in the ultrasensitivity of the system. A small change of the concentration of the input (AMP) can be converted into a much larger change in the final output (kinase activity) (Hardie *et al*., 1999). This excessive sensitivity is very important in the ability of AMPK to maintain the energy state of the cell within narrow limits. AMPK is therefore activated by the metabolic stresses that either inhibit ATP production (e.g. hypoxia, hypoglycemia, oxidative stress or nutrient deprivation) or accelerate ATP consumption (e.g. muscle contraction). After activation, it switches on the catabolic pathways that generate ATP, while switching off the pathways leading to biosynthesis (Figure II) (Hardie *et al*., 2006).

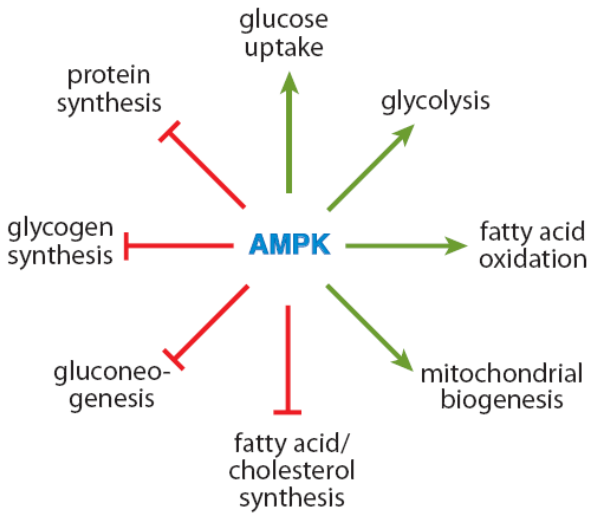


FIGURE II. **Key processes that are regulated by AMPK**. A green arrow indicates activation, whereas a red line with a bar at the end indicates inhibition.

The AMPK is an evolutionarily conserved enzyme and its regulation was shown to be similar between mammals and insects. It was shown that AMP does activate the AMPK orthologue from *Drosophila* (Pan & Hardie, 2002).

The stress events are accompanied by a rapid release of extracellular nucleotides from damaged tissues or activated endothelial cells and platelets (Gordon *et al*., 1986). Da Silva *et*

al. (2006) demonstrated that extracellular nucleosides (ATP, ADP, and UTP) and adenosine independently induced phosphorylation of AMPK in human umbilical vein EC (HUVEC). It was proved that this activation was not mediated by adenosine receptor but required adenosine uptake by equilibrative nucleoside transporters followed by adenosine conversion to AMP (da Silva *et al*., 2006).

Extracellular ATP and ADP and other nucleoside triphosphates and diphosphates are involved not only in the energetic processes but also function as both autocrine and paracrine signaling molecules. They influence cell growth regulation, programmed cell death, secretory processes and ion channel activity (Ralevic & Burstock, 1998). The ATP may exit the cells by both passive ways (resulting from the loss of plasma membrane integrity) and various active transport mechanisms. Several mechanisms are involved in the release of nucleotides depending on conditions and cell types: ATP binding cassette (ABC) transporters, connexin hemichanels, mitochondrial porins (VDAC) and stretch-activated channels (Lazarowski *et al*, 2003). ATP can be also stored in the synaptic vesicles and upon nerve stimulation it is released into synaptic cleft with other transmitters (Todorov *et al*., 1997).

Many investigations reported that adenosine, adenine nucleotides or related agonists stimulate or inhibit the proliferation of variety of normal and transformed cell types (Rounds *et al*., 1998; Bradley & Bradley, 2001; Ohkubo *et al*., 2007). The inhibitory mechanisms can be mediated by activation of AMPK, which represses energy-demanding processes, such as DNA replication and cell division (Hardie *et al*., 1999). The growth arrest can be also caused by uridine deprivation (Weisman *et al*., 1988; Ohkubo *et al*., 2007). Ohkubo *et al*. (2007) reported that simultaneous addition of uridine completely reversed the AMP-or adenosine-induced cell growth inhibition.

In insect cells the effect of adenosine on cell division and morphology was also observed (Zurovec *et al*., 2002). It was demonstrated that ADGF and ADA proteins stimulate proliferation of Cl.8+or S2 cells by modulating extracellular adenosine level.

2 SPECIFIC AIMS

- To examine the adenosine response of Cl.8+ cells in complete medium by testing their growth properties and mitochondrial membrane potential.
- To investigate whether the extracellular adenosine influences ATP level in Cl.8+ cells.
- To find out whether adenosine stimulates O_2 consumption in Cl.8+ cells.
- To elucidate the role of DmEnt2 adenosine transporter in the cell response to adenosine.

3 MATERIALS AND METHODS

3.1 Reagents

Adenosine, dipyridamole and cyclohexyladenosine (XAC) were purchased from Sigma Aldrich CO. Adenosine powder was kept at 4 °C. Working solution was prepared in MM as 1mM and kept at –20 °C. Dipyridamole and XAC were kept at –20 °C both as a powder and a 10mM diluted stock. Dipyridamole was diluted in DMSO and XAC in PBS containing 0.1 M NaOH.

Adenosine, dipyridamole and XAC were used at final concentrations of 25-200 μM, 0.1-100 μM and 0.1-100 μM, respectively.

3.2 Cell Culture

Drosophila imaginal disc cells (Cl.8+) were maintained in complete medium (CM) which was Shields and Sang medium (Shields & Sang, 1970, Sigma Aldrich CO), supplemented with 2 % FBS, 2,5 % fly extract, 125 U/l insulin, 1 % penicilin/streptomycin (10000 U/10 mg) and $KHCO_3$ (0,5 g/l). Minimal medium (MM) was Shields and Sang medium lacking yeast extract, supplemented with L-leucine, L-methionine, fumaric acid, folic acid, myo-inositol, pyridoxine, riboflavin, and thiamine. Media were filter sterilized (0.2 μm porosity, Nalgene). Cl.8+ cells were passaged every 2 days. Confluent cells at passages 90-110 were used for all experiments.

3.3 Cell Treatment

Before each experiment the cells were harvested from 6 or 10 cm dishes by washing with CM and their concentration was determined by counting using haemocytometer. Cells were diluted in CM to certain concentration and plated on the wells or plates. After 4 hours of incubation, the cells were washed with PBS and CM (in case of control) or CM containing adenosine to minimize the effect of cellular adenosine deaminases. In the experiments with ENT or AdoR inhibition the cells were preincubated with appropriate drugs for 20 minutes. All experiments were performed in CM. Although the CM composition is complex and not strictly defined due to its additives (serum, fly and yeast extract), it provides optimal growth conditions for Cl.8+ cells.

3.4 Cell Growth Rate Analysis

The cells were plated on six-well plates at 4×10^5/ml, pretreated as described in *3.3* and an appropriate medium was added. The proliferation of the cells was measured by direct counting of cells by using digital photographs of identical areas taken every 24 hours in a period of 4 days. Five treatments were examined – 50 μM adenosine (Ado), 100 μM Ado, 200 μM Ado, 10 μM dipyridamole + 100 μM Ado and control (untreated cells).

3.5 Measurement of Mitochondrial Membrane Potential ($\Delta\psi_m$)

Mitochondrial membrane depolarization was determined by using a fluorescent dye TMRE (tetramethylrhodamine ethyl ester perchlorate) (Invitrogen). Changes in $\Delta\psi_m$ cause redistribution of TMRE across the inner mitochondrial membrane. This is detected as variations in the measured fluorescence intensity of the dye. The accumulation of TMRE in mitochondria depends on the $\Delta\Psi_m$ and cannot bind to mitochondria with impaired membranes. As a results of mitochondrial membrane depolarization, a shift to the left in the emission spectrum by impaired cells can be detected. Detection is performed by flow cytometer.

The cells were seeded in a concentration of 2×10^6/ml into a six-well plate, pretreated as described in *3.3* and incubated with 200 μM adenosine for 24 hours. After incubation, the cells were harvested in the 0.5 ml volume and TMRE was applied. The TMRE working solution was prepared just before each experiment and added to cells in a final 600 nM concentration. The cells were further incubated in the dark for 15 min at 24 °C.

Samples were examined by flow cytometer EPICS XL-MLC (BECKMAN COULTER) equipped with 15 mW argon laser with excitation capacity of 488 nm. Obtained data were analyzed by WinMDI software (BECKMAN COULTER).

3.6 Measurement of ATP

ATP was measured by the kit The CellTiter-Glo® Luminescent Cell Viability Assay (Promega). This method was originally developed to determine the number of viable cells. The principle of this viability test is based on the amount of ATP, which is high in metabolically active cells. Based on the recommendation of the supplier, the same kit can be applied for the measurement of relative ATP amount in cell culture. The ATP amount in cells

is directly proportional to generation of luminescent signal (stable glow-type luminescent signal) by firefly luciferase enzyme. Figure III represents the principle of luciferase reaction.

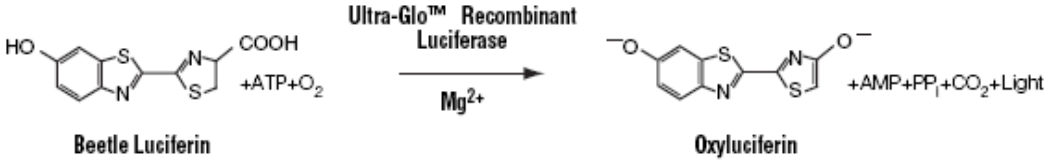


FIGURE III. Mono-oxygenation of luciferin is catalyzed by luciferase in the presence of Mg^{2+}, ATP and molecular oxygen.

Cells were seeded on 96-wells plates at a concentration of 2×10^6/ml, 100 μl of cell suspension was seeded to each well. The cells were pretreated as described in *3.3* and 100 μl of appropriate medium with or without drug was applied. The cells were further incubated for 14 hours at 25 °C. The incubation times varied in time-course studies (every 2 hours). After incubation, 100 μl of „CellTiter-Glo Reagent" was added and cells were lysed (shaking for 5 minutes). After the lyses the whole volume was transferred to a new 96-well plate (made of white plastic) and incubated for 10 minutes at room temperature for signal stabilization. When studying time-course of ATP generation, strictly fixed periods of incubation were desired. The resulting luminescence was detected by Luminometer Orion II with Simplicity 4 software. Each sample was measured for 1 sec.

The concentrations of adenosine used in the experiments were 25 μM, 50 μM, 75 μM, 100 μM, 125 μM, 150 μM, 175 μM, 200 μM. Concentrations of dipyridamole used in the experiments were 0,1 μM, 1 μM, 10 μM, 100 μM. For time-course studies values were obtained in 2, 4, 6, 8, 10, 12 and 22 hours for 4 concentrations (25 μM, 50 μM, 100 μM, 200 μM). The untreated cells in CM served as a control sample, CM solely was considered as a blank.

3.7 Respirometry

3.7.1 Measurement of O_2 Consumption

Measurement of O_2 consumption was performed using oxygraph (Strathkelvin 782, 2-Channel Oxygen System version 1.0) equipped with precision microcathode Clark-type electrodes

(Laboratory of Dr. Lukes, Inst. of Parasitology). The volume of the respiratory chamber was 0.5 ml.

The cells were grown on 6 cm dishes to density of 2×10^7/ml. The cells were pretreated as described in *3.3* and further incubated with 100 μM adenosine for one or two hours. After incubation with adenosine the cells were harvested to a final volume of 0.7 ml. 0.5 ml was used as a sample for the respiratory chamber and the remaining volume was kept on ice for cells number determination by counting using haemocytometer.

Before the measurement the oxygen electrodes were calibrated using sodium hydrosulphite to determine conditions with 0 % oxygen and aerated distilled water to determine conditions highly saturated with oxygen. Water in the chamber stirred. Between particular measurements the respirometric chamber was washed with distilled water. Cell suspension was oxygenized by bubbling and put into the chamber. The O_2 consumption was measured at 25 °C. The respiration was recorded for 5 minutes using Strathkelvin computer software. Recorded data were displayed as a curve representing the course of O_2 consumption.

3.7.2 Data Analysis

Obtained data were analyzed by Strathkelvin software. A certain time interval of respiration course was selected for analysis. The measuring system turned out to be stabilized after 2-3 minutes of measurement, so that the data of 3-5 min of recording were processed. After evaluation, the rate of O_2 consumption was calculated. This result was subsequently corrected for cell density as determined by counting using haemocytometer. Obtained data were expressed as percentage of the control (untreated cells).

3.8 Statistics

The results are presented as means ± SD of 3 independent experiments. Statistics analyses of the data were performed in program Statistica 6.0 (StatSoft Inc) using one-way analysis of variance (ANOVA) followed by the post hoc Tukey's test for multiple sample comparison. Concentration dependent effects were tested by regression analysis. Differences between groups were rated significant at a probability error (P) of <0.05 or 0.01.

4 RESULTS

4.1 The Effect of Extracellular Adenosine on Morphology and Proliferation of Cl.8+ Cells in Complete Medium (CM)

I decided to use the complete growth medium (CM) in all experiments to examine how Cl.8+ cells react to adenosine in optimal growth conditions. Minimal medium (lacking yeast extracts, fly extracts and serum) used in previous studies is chemically defined, but poor nutritionally. We were afraid of the starvation of cultured cells that might cause some stress or altered sensitivity to some treatments.

First of all, it was examined how extracellular adenosine influences the rate of cell division. Therefore growth curves were generated to show, how the cells proliferate during 3 days with or without adenosine treatment. The result indicated that adenosine (Ado) triggers rapid inhibition of cell division (Figure 1). While the growth curve of control cells represented a typical trend of highly proliferating cells, the division of cells in 50 µM Ado was suppressed. Proliferation of cells incubated in 100 µM and 200 µM Ado was highly inhibited. Figure 3a (A-D) and Figure 3b (E-H) show control cells in comparison with the cells treated with various adenosine concentrations after 2 days of incubation. The cells treated with adenosine

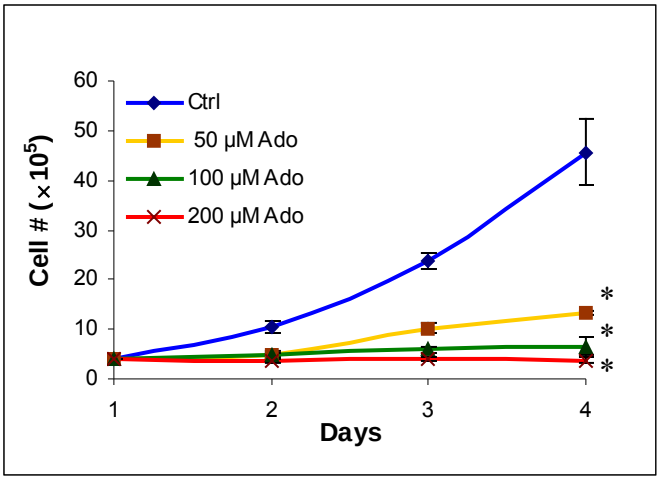


FIGURE 1. Effect of Adenosine on Cell proliferation in Cl.8+ cells. Cells were treated with or without various adenosine (Ado) concentrations and incubated for 3 days. Cells were counted every 24 hours. Values are presented as means ± SD of three independent experiments. *P<0.01 for whole data series compared with corresponding control by Tukey's test.

exhibit block of cell division and altered morphology. They are round and flat, whereas the control cells are elongated and develop pseudopodia (Figure 3a A-D, 3b E-H). We could estimate, that in 50 µM Ado only about one third of the cells kept the original elongated shape

(Figure 3a C, D), while in higher concentrations the original cell shape occurred rarely.. The amount of cells with elongated morphology roughly correlated with the number of proliferating cells (the growth curves in Figure 1 show moderate increase in the number of cells treated with 50 μM Ado within 3 days of incubation). This ability of the cells to overcome adenosine-induced growth inhibition was never observed in Cl.8+ cells growing in the MM (the lack of nutrients or of some protective or survival factors might be stressful for the cells and result in poor growth rate). The CM supplemented with serum and fly and yeast extracts contains some adenosine deaminases and other putative beneficial components that help the cell to manage possible stressful events, such as increased adenosine concentration.

4.2 Equilibrative Nucleoside Transporter (ENT) Is Involved In Adenosine-Induced Cl.8+ Cell Growth Inhibition

It is evident that adenosine has some clear effects on cell physiology (morphological change, growth inhibition). We were further speculated about the mode of this adenosine action. Since the adenosine could influence the cell either via adenosine receptor or by adenosine transporter, we decided to use specific inhibitors of these molecules, known from the mammalian system, to find out whether the cell response could be prevented by any of them.

To test whether adenosine transport is involved in the growth arrest and change of Cl.8+ cell morphology, we decided to use an ENT inhibitor–dipyridamole. Thus, the cells were first preincubated with dipyridamole (10 μM) and further incubated with adenosine (100 μM) and dipyridamole (10 μM) together. Data revealed, that while the proliferation of cells treated with adenosine solely was highly inhibited, the growth curve of cells incubated both with Ado and dipyridamole did not statistically differ from the control (Figure 2). As it is seen from the Figure 3b, our result clearly demonstrates that dipyridamole is able to block the adenosine-induced inhibition of Cl.8+ cell growth (Figure 3b I, J).

Further, we tried to examine the potential role of adenosine receptor in Cl.8+ cell growth inhibition. For this purpose, we selected an AdoR antagonist–cyclohexyladenosine (XAC) based on our reporter assay results (Zurovec & Nothacker, unpublished). However, we were so far unable to evaluate the effect of XAC on Cl.8+ cells due to technical difficulties (XAC requires alkaline pH to be diluted and these conditions were shown to alter adenosine

responce of Cl.8+ cells). Some preliminary results (not shown) did not show any effect of XAC on Cl.8+ cells, but these results need verification.

Based on our available data we conclude that the inhibition of Cl.8+ cell proliferation is completely dependent on adenosine uptake.

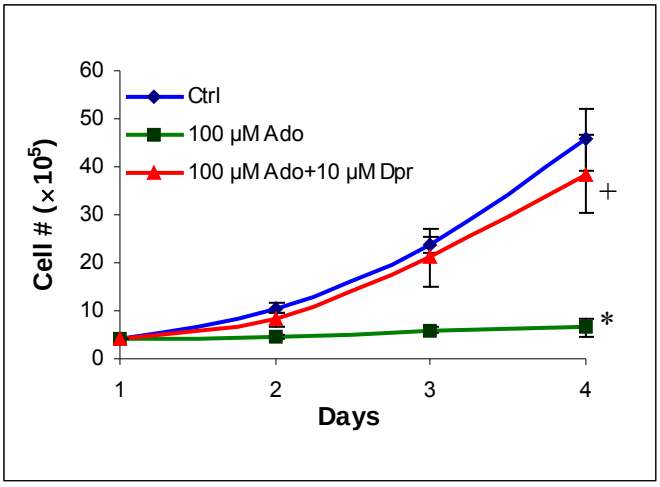


FIGURE 2. Effect of dipyridamole on adenosine-induced Cl.8+ cell growth inhibition. Cells were treated with 100 µM adenosine (Ado) or 100 µM adenosine and 10 µM dipyridamole (Dpr) and incubated for 3 days. Cells were counted every 24 hours. Values are presented as means ± SD of three independent experiments. *P<0.01, +P>0.05 for whole data series compared with corresponding control by Tukey's test.

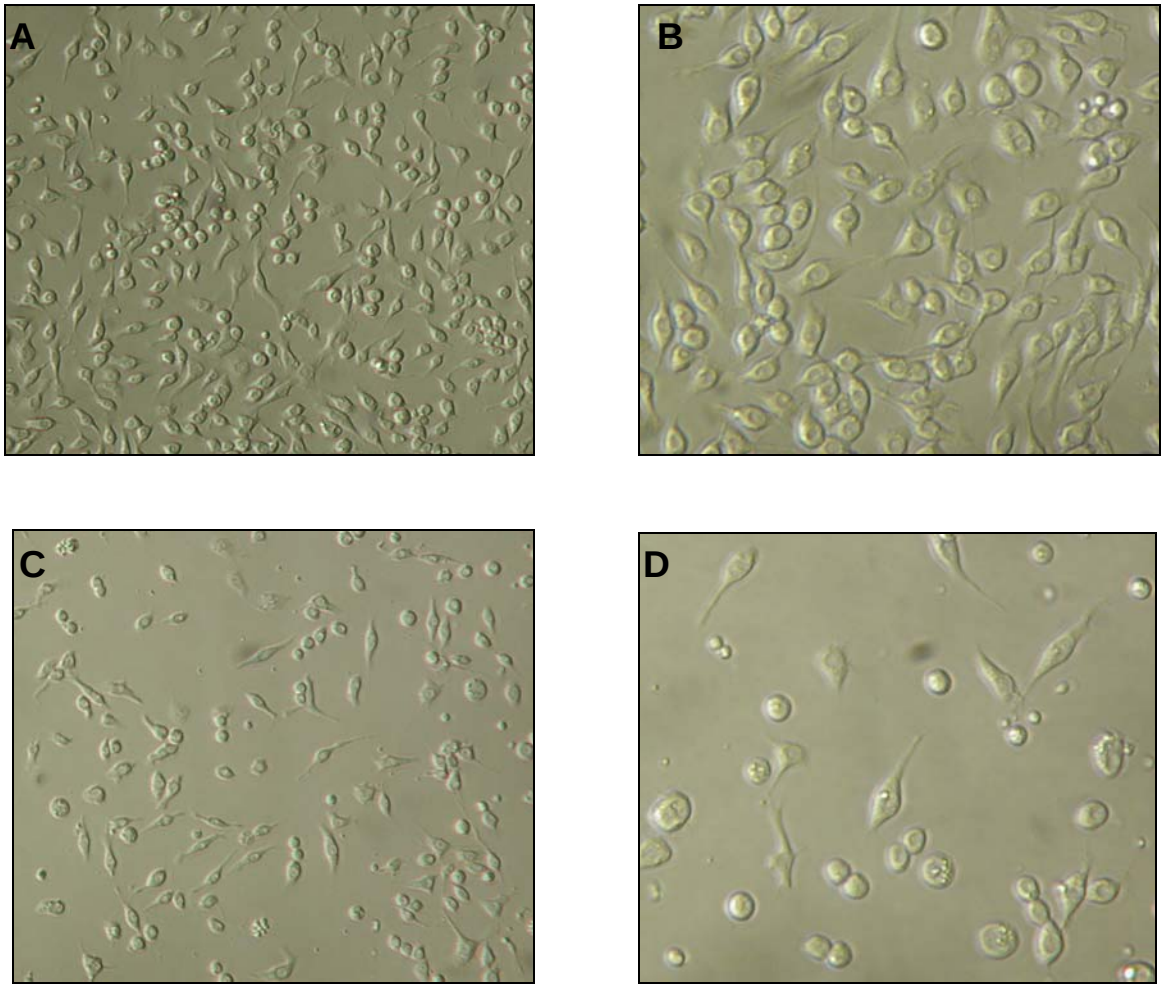


FIGURE 3a. Adenosine effect on Cl.8+ cell proliferation and morphology. The images were obtained after 2 days of incubation with or without adenosine. Untreated control cells (A, B), 50 μM adenosine (Ado) (C, D). A, C indicate magnification 20×; B, D indicate magnification 40×.

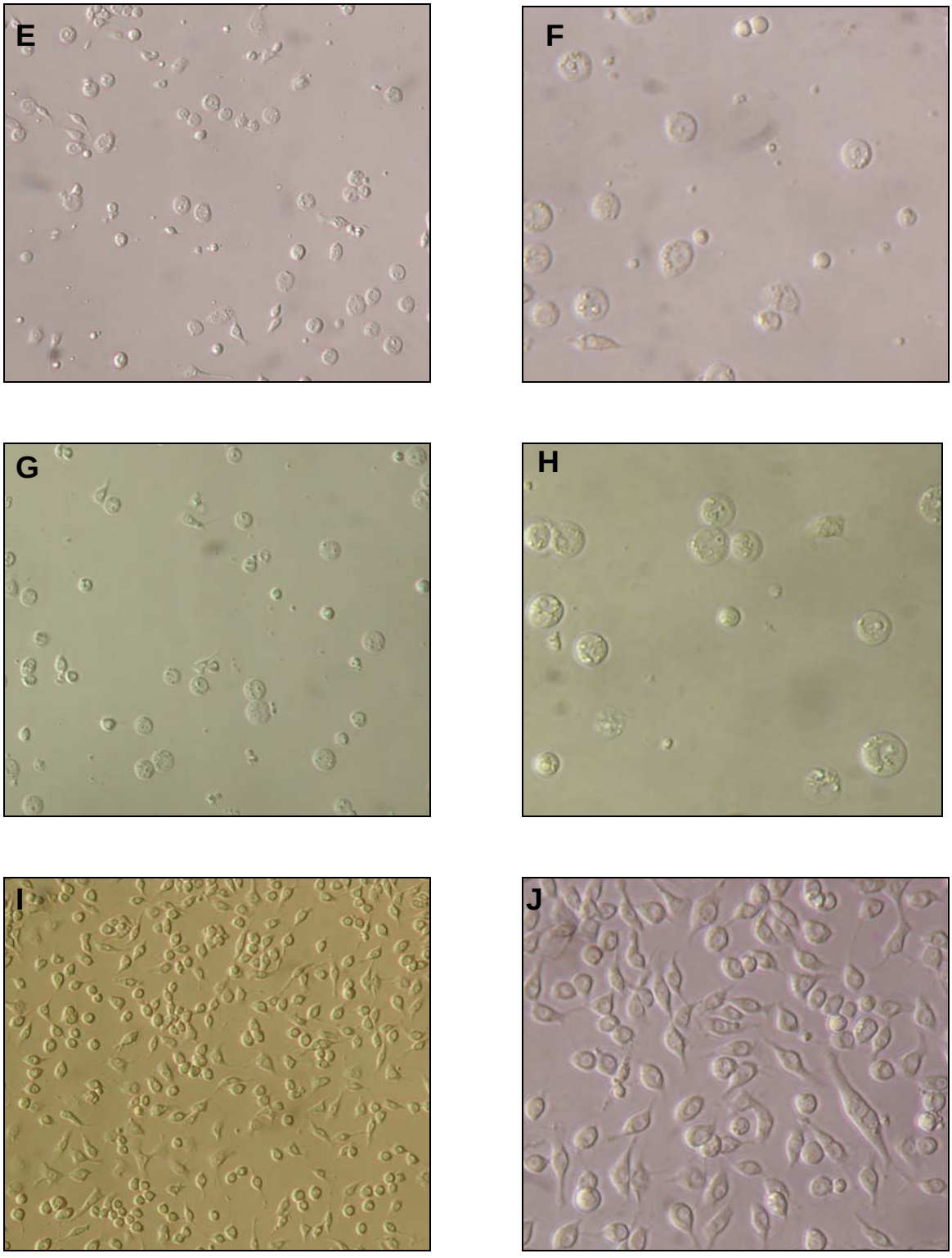


FIGURE 3b. Adenosine and dipyridamole effect on Cl8+ cell proliferation and morphology. The images were obtained after 2 days of incubation with adenosine or adenosine (Ado) and dipyridamole (Dpr). 100μM Ado (E, F) 200 μM Ado (G, H), 100 μM Ado+10 μM Dpr (I, J) , G, I indicate magnification 20×, H, J indicate magnification 40×.

4.3 Mitochondrial Membrane Potentials of Cl.8+ Cells Are Slightly Affected by Adenosine in CM

Since we showed, that adenosine transport is apparently responsible for the morphological change and growth inhibition, we supposed that it could cause some impairments of mitochondrial function or apoptotic events widely observed in these cells in MM (Steinbauerova, 2005). At first we tried to use the MTS test to observe the activity of mitochondrial dehydrogenases. However, this test turned out to be unreliable when using in CM, so we decided to examine the mitochondrial membrane potential ($\Delta\psi_m$) using TMRE staining. Control cells and cells treated with 200 μM Ado were incubated for 24 hours and stained with TMRE.

The results of flow cytometry analysis, using forward scatter (FSC) vs. side scatter (SSC), are represented by specific dot plots (Figure 4A). It can be seen from these dot plot diagrams that the distribution of sorted cells do not significantly differ between the samples examined. Figure 4B represents a histogram showing a specific TMRE fluorescence peak that

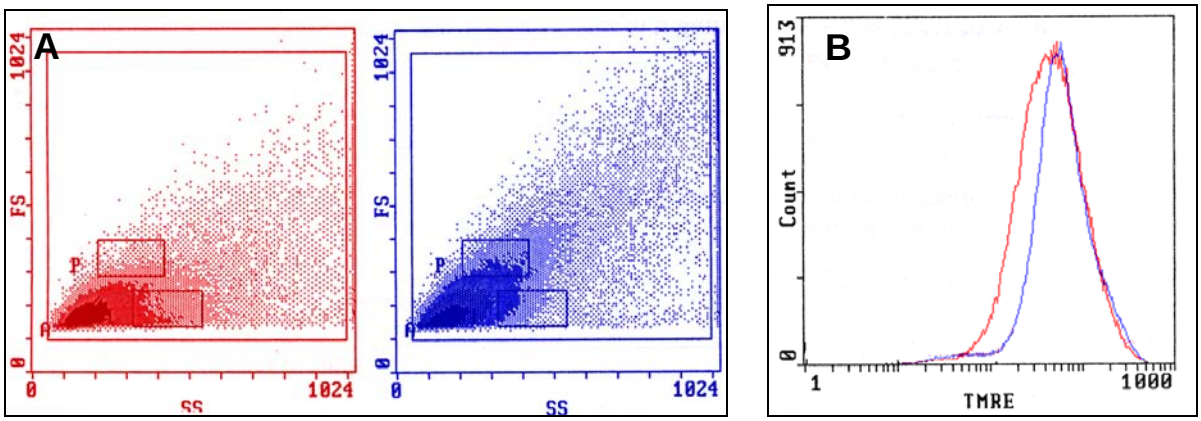


FIGURE 4. Effect of adenosine on membrane potential in mitochondria of Cl. 8+ cells. The cells were incubated with or without 200 μM Ado for 24 hours, after they were stained by TMRE and examined by flow cytometery. (A) Representative dot plots showing the whole cell population scanned by forward scatter (FS) and side scatter (SS). Larger cells are represented as higher values along the y-axis, while the intensity of cellular granularity is represented by x-axis. The red plot indicates adenosine-treated cells, the blue plot indicates untreated control cells. (B) Representative histogram obtained after TMRE staining, x-axis indicates fluorescence intensity, y-axis indicates number of events (cells). Red and blue color indicate adenosine-treated cells and untreated cells, respectively.

the state of $\Delta\psi_m$ corresponding to the entire analyzed population of treated or untreated cells. Surprisingly, these data also showed that adenosine-treated population is quite similar to the control. Both populations exhibit a single peak that is typical for healthy cells. The adenosine-treated cells exhibit only a very slight (8%) shift of the fluorescence peak to the left, which indicates only a subtle attenuation of the mitochondrial membrane potential ($\Delta\psi_m$).

Since we did not confirm previous results demonstrating apoptotic changes after 24 hours in Cl.8+ cells grown in MM described by Steinbauerova (2006), we suggest, that although adenosine is able to trigger a rapid change in cellular growth and morphology in CM, it can perform it without causing apoptosis or with its substantial delay. This implies, that adenosine could have primarily different function in Cl.8+ cells. However, this function might be observed under optimal growth conditions.

From the data obtained it can be assumed that the mitochondrial membranes are normally functional after adenosine induction.

4.4 Effect of Adenosine on Relative ATP Amount in Cl.8+ Cells

Although the TMRE staining did not bring any large difference in mitochondrial membrane polarity, we decided to examine another parameter of mitochondrial function and cell viability – the ATP production. We measured the amount of ATP in cell lysates based on the level of detected luminescence (see section *3.6*; Materials and Methods). The measurements revealed that adenosine increases the ATP amount in Cl.8+ cells in a dose-dependent manner (Figure 5A). General trend of the concentration-dependent effect was evaluated rather as polynomic than linear (Figure 5B, 5C). The proportional increase was detected from 25 μM to 150 μM Ado induction. For instance, 100 μM Ado induced the increase of ATP amount about two times more than 50 μM Ado. Thus, in this range the trend in the dose-response could be considered both linear and polynomic (Figure 5D, 5E). The obtained data support our suggestion that mitochondria are active after adenosine treatment of Cl.8+ cells. Adenosine was found to induce ATP generation and the ATP increase is dose-dependent.

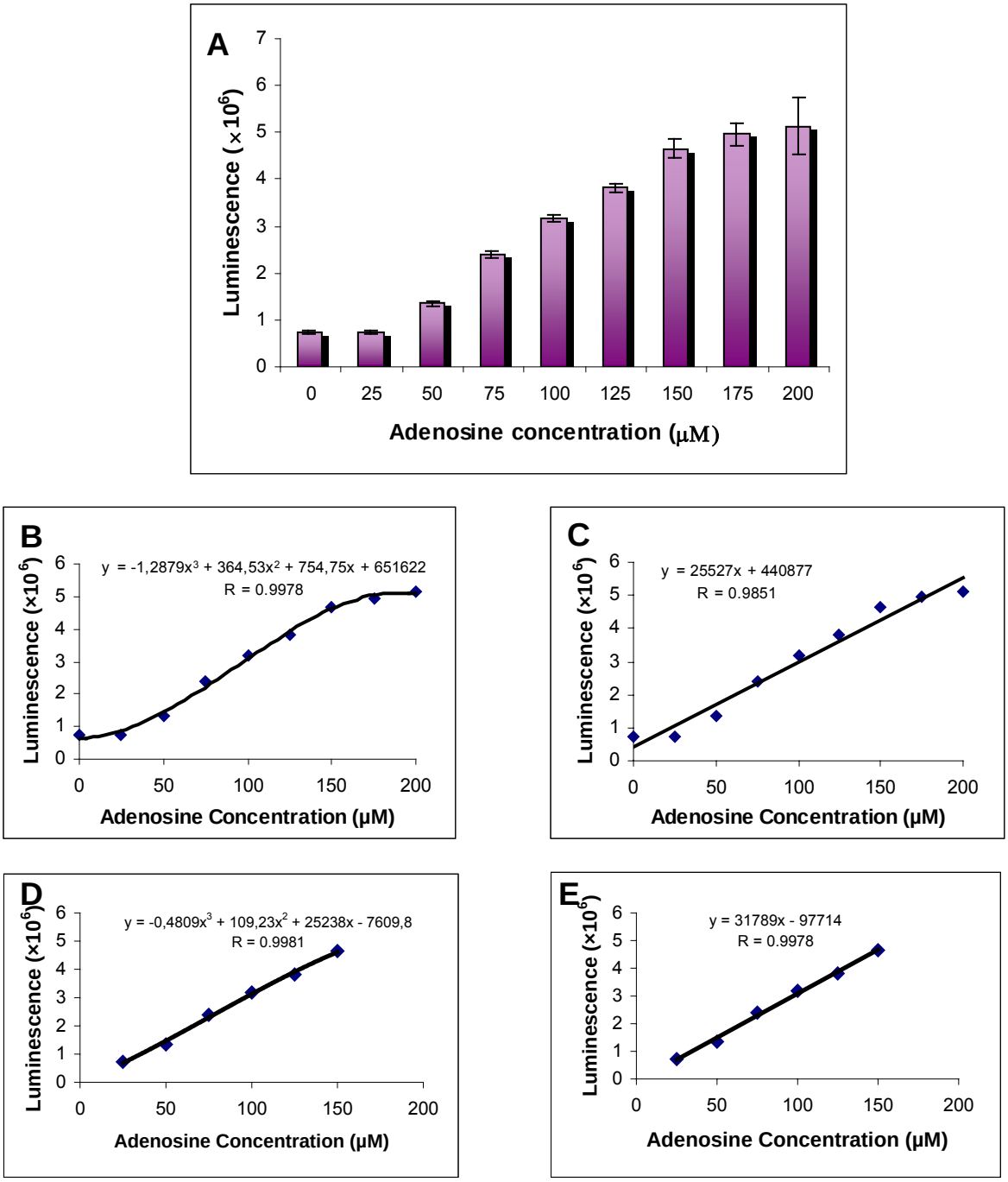


FIGURE 5. Effect of adenosine on relative ATP amount in Cl.8+ cells. Cells were incubated for 14 hours with certain adenosine concentrations. Samples were measured for the level of luminescence corresponding to the relative amount of ATP present in cell lysate. (A) Each column represents mean ± SD of three independent experiments. (B-E) Evaluation of concentration-dependent effect by regression analysis. Two types of regression were compared: polynomic (R = 0.9978) (B) and linear (R = 0.9851) (C) D and E indicate regression curves of 25 µM - 150 µM Ado induction range: polynomic (R=0.9981) (D) and linear (R=0.9978) (E).

We further examined the time-course of adenosine-induced ATP increase (Figure 6). It was found, that Cl.8+ cells started to generate ATP within half an hour after the treatment (data not shown) and the gradual ATP increase continued 4 or 6 hours after treatment (depending on adenosine concentration). After this period, the level of ATP stopped to increase, kept its level and did not further change with time. After 22 hours of incubation only a moderate decrease of ATP amount was observed. It is obvious from the graph (Figure 6) that the maximal ATP level after such "stabilization" is also dependent on adenosine concentration. The more adenosine was added, the higher level of ATP was reached.

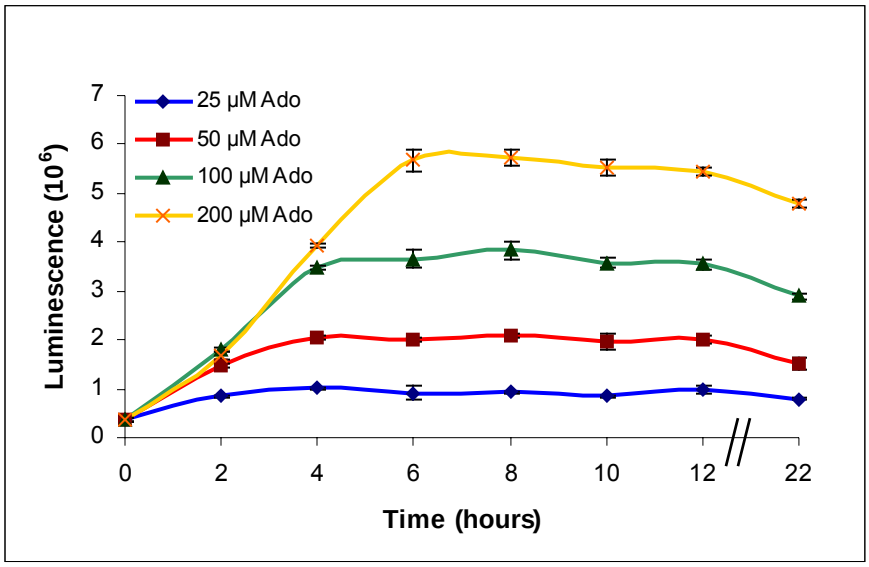


FIGURE 6. Time-course study of adenosine induced increse in relative ATP amount in Cl.8+ cells. The cells were incubated in CM containing certain adenosine (Ado) concentrations (25 µM, 50 µM, 100 µM, 200 µM). Cells were checked for the level of luminescence (corresponding to ATP amount) in regular 2 hours-intervals. Each point represents mean ± SD of three independent experiments.

After this observation, we were interested in the mechanism that keeps the stable level of ATP. Since it is not known how stable is the adenosine in the CM, we treated the cells with the "preconditioned" CM containing 100 µM Ado (the CM with adenosine was incubated for 4 hours with a monolayer of Cl.8+ cells, than the preconditioned medium was collected for further use – see section *3.3* (Materials and Methods). Whereas the regular adenosine treatment caused highly significant increase of ATP, the CM with adenosine conditioned for 4

hours in another culture was unable to trigger any ATP increase (Figure.7). This experiment showed that after 4 hours of incubation the level of adenosine in the CM is quite low.

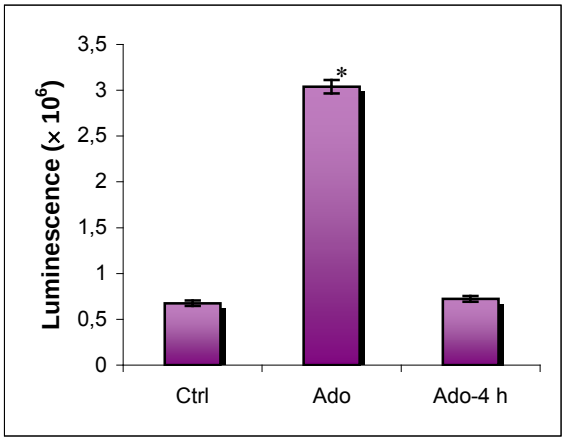


FIGURE 7. Effect of CM with adenosine preconditioned for 4 hours. Compairing of ATP amount after 14 hours of incubation by untreated cells incubated in CM (ctrl), cells incubated in CM+100 μM adenosine (Ado) and cells incubated in CM+100 μM Ado that was previously "preconditioned" for 4 hours by untreated cells (Ado-4 h). Each value represents mean ± SD of 3 independent experiments. ∗p<0.01, compaired with untreated control by Tukey's test.

The fact that the cells need permanent adenosine presence for the stimulation of ATP production was proved by another experiment. Figure 8 shows the test, in which the medium with adenosine was removed from cell culture after 2 hours of incubation and replaced with the fresh CM without adenosine. The samples with media exchange did not exhibit any further ATP increase after 4 or 10 hours of incubation, whereas in samples treated without media exchange, the ATP level further grew.

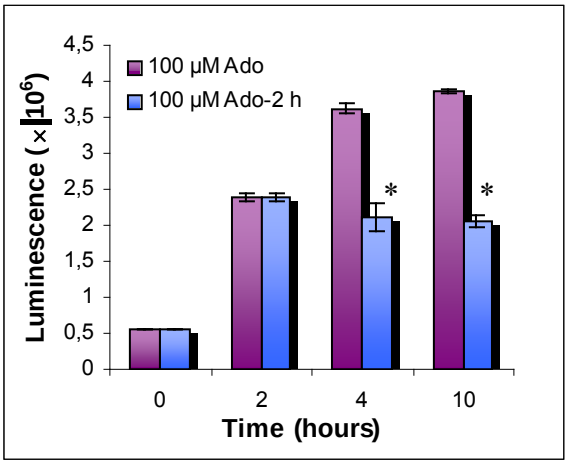


FIGURE 8. Effect of adenosine removal after 2 hours of incubation in adenosine supplemented medium. Cells were treated with 100 μM adenosine (100 μM Ado) for whole period of experiment duration or the medium with Ado was replaced after 2 hours with CM without Ado (100 μM Ado-2 h). ∗p<0.01, compared with corresponding values of classical treatment by Tukey's test.

All these results show that extracellular adenosine regulates the level of ATP in Cl.8+ cells, the amounts of the generated ATP are proportional to the adenosine concentration and the intracellular level of ATP is stable for several hours. Interestingly, the extracellular adenosine must be present with cells until the "plateau" in the ATP synthesis is reached.

4.5 Adenosine Uptake Is Essential for Adenosine Induced ATP Increase in Cl.8+ Cells

Since we examined previously that adenosine transport is important in the morphological and growth responses of Cl.8+ cells to increased level of adenosine in the CM, we wanted to examine whether the adenosine transport also influences the ATP stimulation. As in previous experiments, the ENT inhibitor dipyridamole was used. As it is shown in Figure 9a, the

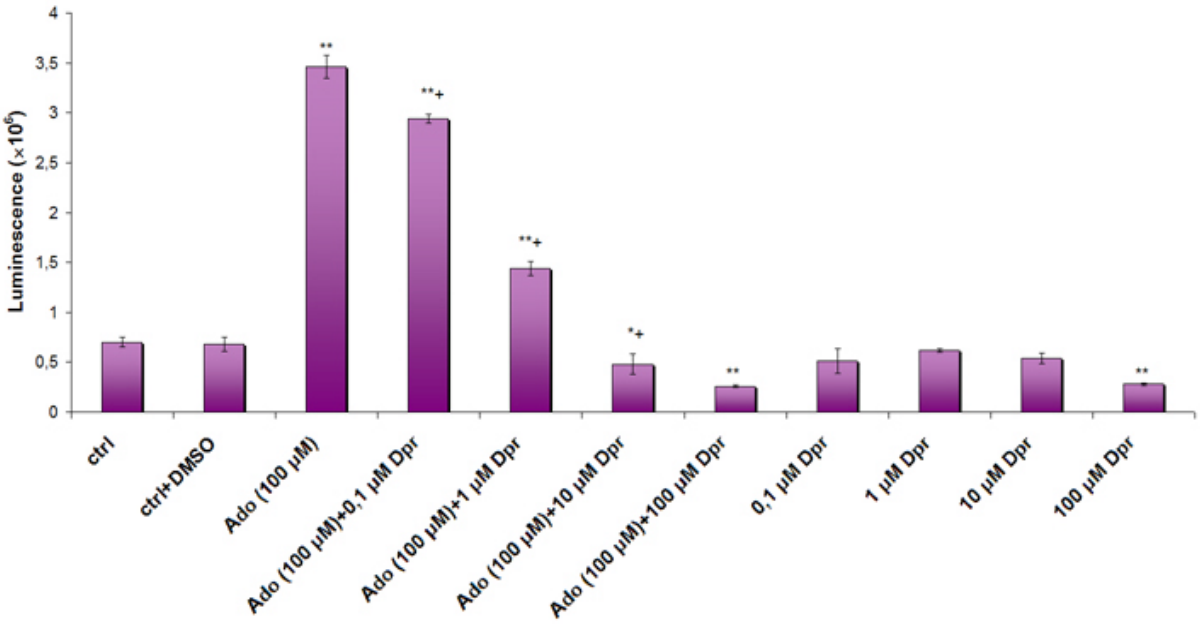


FIGURE 9a. **Effect of dipyridamole on adenosine-induced ATP increase**. The cells were preincubated with or without various concentrations of dipyridamole (Dpr) for 20 min, and then they were further incubated with 100µM adenosine (Ado) or 100µM adenosine + certain concentration of dipyridamole for 14 hours. Each column represents mean ± SD of three independent experiments. **P<0.01, *P<0.05 compared with control by Tukey's test, +P<0.01 for Ado+Dpr compared with Ado alone by Tukey's test. The dose-dependent effect was tested by regression analysis, type of regression was evaluated.

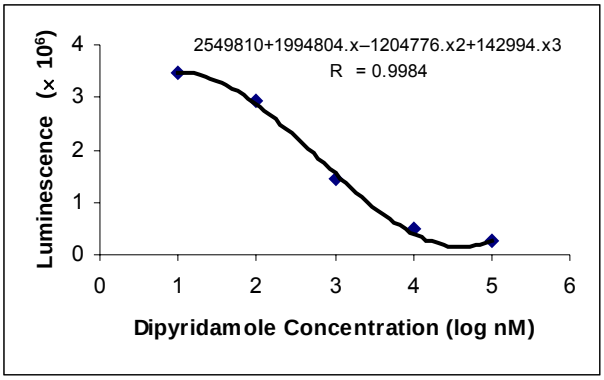


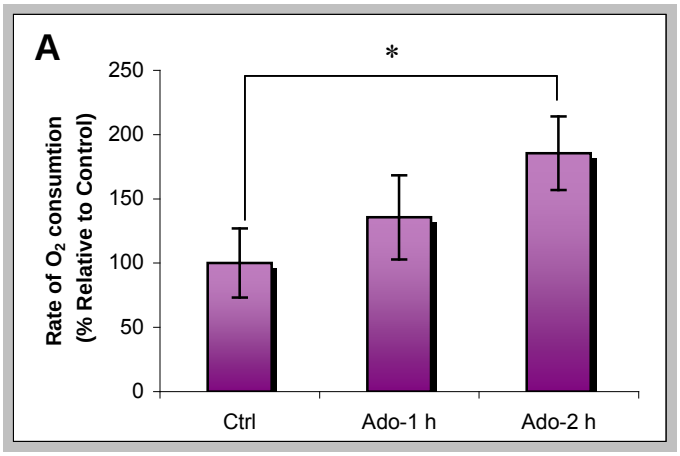
FIGURE 9b. Inhibitory effect of dipyridamole on adenosine-induced ATP increase. The dose-dependent effect was tested by regression analysis, type of regression was evaluated as polynomic (R=0.9984).

results confirmed that dipyridamole was able to inhibit the adenosine-induced ATP increase. Dipyridamole concentration as low as 0.1 μM significantly attenuated the increase of ATP amount induced by 100 μM adenosine. 1 μM dipyridamole prevented the ATP increase of more than 50 percent. The level of ATP after treatment with 10 and 100 μM dipyridamole was even lower than ATP level in control cells (Figure 9a). As expected, the dipyridamole inhibition of ATP synthesis was dose-dependent (Figure 9b). These results clearly demonstrate that adenosine transporter is essential for adenosine-mediated ATP increase.

4.6 Effect of Adenosine on O_2 Consumption in Cl.8+ Cells

To prove that the synthesis of ATP is generated by higher rate of oxidative phosphorylation, we should be able to detect higher oxygen (O_2) consumption.

Using an oxygraph, we measured the rate of O_2 consumption in control and adenosine-treated cells As it is shown in Figure 10, our experiments clearly showed that the cells treated with adenosine exhibit a higher rate of O_2 consumption. From 5 min-recording, data of last 2 minutes were selected for evaluation (see section *3.7.2*; Materials and Methods). Data obtained from 4^{th} and 5^{th} minute of recording (Figure10A and 10B respectively) show, that the cells incubated in 100 μM adenosine for 2 hours had significantly higher rate of oxygen consumption than the untreated cells. Our results confirm that adenosine has a stimulatory effect on aerobic respiration in Cl.8+ cells.



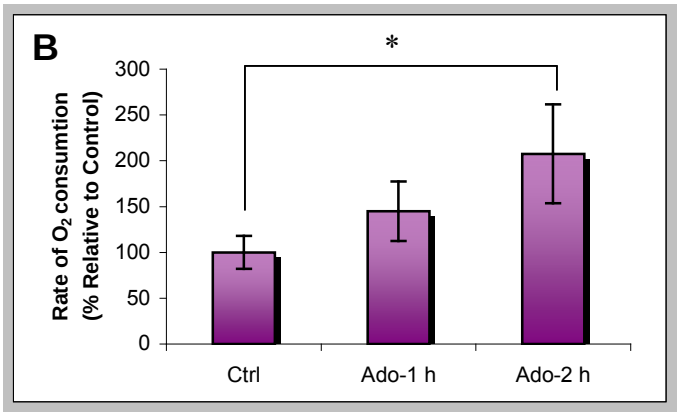


FIGURE 10. Effect of adenosine on O_2 consumption in Cl.8+ cells. The cells were treated with 100 μM adenosine (Ado) for 1 hour (Ado-1 h) or 2 hours (Ado-2 h). After incubation they were harvested to the density of 2×10^7/ml and put to respiration chamber. Each recording took 5 minutes. A and B indicate 4th and 5th minute of recording, respectively. Ctrl indicates untreated control cells. Values are presented as means ± SD of three independent experiments. **P*<0.05 compared with control by Tukey's test.

5 DISCUSSION

Here we report that extracellular adenosine has an important effect on oxidative phosphorylation and mitochondrial energetics in *Drosophila* imaginal disc cells (Cl.8+) *in vitro*. The addition of extracellular adenosine to the growth media leads to the increase in intracellular ATP levels in a dose-dependent manner and to the stimulation of O_2 consumption rate. These effects are simultaneously accompanied by morphological change, inhibition of cell proliferation but without causing immediate apoptosis detected previously in experiments using minimal medium. The majority of evidence indicates that equilibrative nucleoside transporter ENT2 rather than the adenosine receptor is responsible for the mediation of these adenosine effects.

5.1 Adenosine Effect on ATP Generation in Cl.8+ Cells

Our data show that the elevated extracellular adenosine induced the increase of ATP in a dose-dependent manner in Cl.8+ cells grown in complete medium (CM). Previous studies examining the adenosine effects in minimal media (MM) reported morphological change, growth arrest, decrease of mitochondrial staining and eventual death of the cells by apoptosis (Zurovec, 2002; Steinbauerova, 2005). In contrast, we did not observe any apoptosis in Cl.8+ cells in the CM after adenosine treatment with concentrations up to 200 μM within 24 hrs. The TMRE mitochondrial staining, reflecting the mitochondrial membrane polarity of adenosine treated cells in CM, showed only 8 % shift of the peak median compared to controls. We observed that in CM conditions, adenosine induced morphological change and growth inhibition of Cl.8+ cells, but it did not induce immediate apoptosis. After 24 hours of incubation, some cells were able to overcome their growth arrest, returned to the cell cycle and continued growing (the number of growing cells was higher at lower adenosine concentrations, e.g. 50 μM). In the experiments of Steinbauerova (2006), the apoptosis of Cl.8+ cells usually occurred after 24 hours after adenosine treatment, which was much later than in cells treated with staurosporine, cycloheximide or UV irradiation (Steinbauerova, 2006). It is possible that since the effect of extracellular adenosine also triggers the massive ATP synthesis in Cl.8+ cells, the adenosine-induced cell death of these cells in poor growth media is a secondary consequence of lack of nutrients rather than a primary adenosine effect. Alternatively, some putative component present in CM (e.g. adenosine deaminase or IDGF)

attenuate the intensity of adenosine action. In contrast, triggering of the ATP production in the rich CM, would only cause morphological change and growth inhibition.

The contribution of adenosine to the elevation of adenine nucleotide pool was already observed in rat skeletal muscle cells and endothelial cells (Hellsten & Frandsen, 1997). They showed that over 95 % of the adenosine label was present in ATP, ADP and AMP after 30 minutes of incubation with radioactive adenosine. Adenosine-induced increase in ATP level was also demonstrated in porcine vascular smooth muscle (Barron & Gu, 2000), where the incubation of porcine arterial strips for 90 min with 0.5 mM adenosine resulted in significant increases in ATP, ADP and AMP levels. It was also proved in endothelial cells, that adenosine and adenine nucleotides replete adenosine triphosphate after oxidant injury by adenosine uptake (Andreoli et al.,1990). The adenosine-induced ATP increase described in this thesis is also supported by results from Tichy (2006), who observed a massive concentration of ATP after 100 μM adenosine treatment.

The time-course study of adenosine effect on ATP increase in Cl.8+ cells revealed that the level of ATP stopped to increase and stabilized after a specific time period of incubation with adenosine. For instance, the incubation in 100 μM adenosine resulted in a gradual ATP increase during the first 4 hours. After 4 hours of incubation the level of ATP became stabilized at certain level and did not change during several hours of additional incubation. Furthermore, we suggest that the level of ATP stabilization is determined by the time of adenosine degradation or depletion. The CM medium with adenosine, previously incubated with cells for 4 hours, was unable to trigger ATP increase when added to a fresh plate with cells. This suggests that adenosine was degraded or depleted after 4 hours of incubation.

In another experiment we examined, whether the growth media replacement and removal of adenosine before the "ATP plateau" is reached would affect the final ATP level. The cells were incubated with adenosine for 2 hours. Subsequently, the medium with Ado was replaced by the fresh CM without any extra adenosine. Interestingly, the resulting level of ATP stopped growing and the cells kept the stable level of ATP they reached at the moment of the media exchange. It means that there was still some adenosine in the medium after two hours of incubation and its presence was needed for the ATP synthesis stimulation. This result is more consistent with the idea that the adenosine (in the conditioned medium, during the 4 hours period) is rather depleted by the cells than deaminated.

Our data are rather preliminary at this moment and will require the exact measurement of AMP, ADP and ATP ratios in time. So far, our results are consistent with the idea that the adenosine transported into the cell might influence the intracellular levels of AMP, ADP and ATP and shift the energy balance to ATP synthesis. After reaching a new balance between intracellular ATP/AMP, the level of ATP would stay stable for several hours even after removal of extracellular adenosine.

5.2 Adenosine Stimulates O_2 Consumption in Cl.8+ Cells

We verified that the ATP generation in Cl.8+ cells is accompanied by an increase in O_2 consumption. After two hours of incubation with 100 μM adenosine, the rate of O_2 consumption was significantly higher in comparison to untreated control cells. To our knowledge this is the first report of the adenosine stimulation of aerobic respiration in insect cells.

Similar findings were obtained in experiments with mammalian tissue cultures (Barron & Gu, 2000). The measurement of O_2 consumption by porcine carotid artery strips revealed that 0.5 mM adenosine stimulated O_2 consumption by 52±10%. Moreover, this result was accompanied by the increase of ATP production. Extracellular adenosine, transported into the myocyte, is phosphorylated to AMP and hence to ADP and ATP (Sparks et al., 1986). The increase of ADP relative to ATP would decrease the ATP:ADP ratio, which is a major factor controlling the O_2 consumption (Heineman & Balaban, 1990). The resulting increase in O_2 consumption would even further stimulate formation of high-energy phosphate (Barron & Gu, 2000).

If the adenosine would increase the O_2 demand as described above, it would increase hypoxia and ischemia and would not be protective. However, the adenosine has another effect in mammals, connected with the function of adenosine receptors. It stimulates the cerebral and myocardial blood flow during hypoxia. (Tabrizchi & Bedi, 2001)

It has been well established in studies on mammalian cardiovascular and neural systems that adenosine has cardioprotective (Shryock & Belardinelli, 1997) and neuroprotective (Pedata *et al*., 2005) effects. The myocardial oxygen consumption increases as much as fivefold during exercise when heart rate and myocardial contractility are elevated. The increase in myocardial oxygen consumption can only occur by a similar increase in oxygen delivery provided by

augmented coronary blood flow. A2A adenosine receptor mediates coronary vasodilation during exercise, which increases the oxygen supply. (Bache *et al.,* 1988; Erga *et al*., 2000) Moreover, the limited availability of oxygen under hypoxia prevents major ATP supply through the electron transport pathway, hypoxia leads to the decrease in intracellular ATP and increases in its degradation products, AMP and adenosine. Normally, adenosine kinase can decrease adenosine levels by phosphorylation of adenosine; however, the hypoxia-associated inhibition of adenosine kinase enhances the accumulation of adenosine under hypoxia (Sitkovsky & Ohta, 2005).

Adenosine effect on oxygen balance in insects remains unclear. However, one surprising result was obtained in Manduca sexta (Lepidoptera). After injection of adenosine to diapausing pupae, intensive breathing movements were observed for 24 hours, while controls (injected with insect Ringer's solution) did not exhibit any change (Slama, unpublished results). Therefore we suppose, that adenosine might also serve as a signal for the increase of oxygen supply in insects. We speculate that the mechanisms of oxygen balance regulation are dependent on adenosine release and combine action of adenosine receptors and transporters. We expect this mechanism is conserved among phyla.

5.3 Adenosine Uptake and Potential Metabolic Pathway Involved

Our experiments with dipyridamole, which is an ENT inhibitor, suggested that adenosine transport is responsible for the ATP increase, as well as for the morphological change and growth arrest of Cl.8+ cells. The results revealed that dipyridamole is able to prevent the ATP increase in a dose-dependent manner. In previous experiments performed by Steinbauerova (2006), the transfection of Cl.8+ cells with RNAi construct knockouting the ENT2 expression (which has the highest expression of all *Drosophila* ENTs in Cl8+ cells) lead to the rescue of Cl.8+ cells in minimal medium containing adenosine. Both these results strongly suggest that adenosine transport plays a key role in the response of Cl.8+ cells to the extracellular adenosine.

The involvement of adenosine transport by ENT in adenosine response was shown in a number of mammalian cell types (Da Silva *et al*, 2006; Wang & Ren, 2006; Andreoli *et al*., 1990), where the reported importance of adenosine transport was also proved by application of the ENT inhibitor – dipyridamole. Furthermore, it was found, that adenosine, entering the cell

via specific transport, is subsequently converted to AMP via adenosine kinase. AMP is a strong activator of an energetically very important enzyme – the AMP-activated protein kinase (AMPK) (Hardie, 2004, da Silva *et al*., 2006). Mechanism of AMPK activation is highly conserved at least between mammals and insects (Hardie, 2004). Since its activation was found to stimulate ATP generation or inhibit cell proliferation (Hardie, 2004), the AMPK could be a potential candidate for future studies on Cl.8+ cells. In our preliminary experiments performed in collaboration with Radimerski (Basel, Switzerland) we found that adenosine induces S6-kinase in Cl.8+ (Zurovec and Radimerski, unpublished). Since the S6K is an important downstream target of AMPK, these results are consistent with the proposed pathway of adenosine signal in Cl.8+ cells causing the energetic changes.

Although the adenosine uptake was identified as an essential factor in the adenosine-mediated stimulation of ATP synthesis in Cl.8+ cells, the potential role of adenosine receptor may not be excluded. Da Silva *et al* (2006) and Ohkubo *et al.* (2007) disproved the involvement of adenosine receptor in adenosine-mediated effect on AMPK phosphorylation and cell growth inhibition (respectively) using specific AdoR antagonists, but it is necessary to test this alternative also in Cl.8+ cells. This will be the subject of further investigations.

So far it is not clear, what is the physiological explanation for the Cl.8+ cell adenosine response, revealed in this study. The matter of our interest is also to examine adenosine effect on other *Drosophila* cell types. Preliminary results show that the adenosine response might differ among different cell types. Whereas *Drosophila* embryonal S2 cells exhibit similar behavior as the Cl.8+ cells, a haemocytic cell line Mbn-2 was found to be resistant to adenosine, and no increase in ATP amount was observed after adenosine treatment in these cells (data not shown). This could be explained by a high level of expression of adenosine deaminases in these cells observed by Zurovec *et al*. (2002), or by a low level of adenosine transport. This topic must be examined in more detail.

6 CONCLUSIONS

- Adenosine treatment of Cl.8+ cells in complete medium led to growth inhibition and morphological change.
- Examination of mitochondrial membrane potential $\Delta\psi_m$ (using the probe TMRE and flow cytometry) revealed a very minor mitochondrial depolarization .
- Measurement of intracellular ATP concentration revealed that adenosine caused ATP increase in dose-dependent manner and that the ATP level became stabilized and was kept constant for several hours.
- Measurement of O_2 consumption showed that adenosine treatment stimulated the rate of aerobic respiration in Cl.8+ cells.
- Equilibrative nucleoside transporter (ENT2) was identified as a crucial factor in both growth inhibition and ATP increase.

7 REFERENCES

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